Author's response to reviews

Title: Early over expression of messenger RNA for multiple genes, including insulin, in the Pancreatic Lymph Nodes of NOD mice is associated with Islet Autoimmunity

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Author's response to reviews: see over
Discretionary Revisions

Reviewer’s comment:
Based on their former finding that the presence of insulin autoantibodies (E-IAA) predicts early diabetes onset in NOD mice, Regnault et al. analyzed gene expression profiles in pancreatic lymph nodes (PLNs) of E-IAA(+) and E-IAA(-) 5 weeks old NOD mice to identify genes implicated in the early steps of the autoimmune process. They found gene expression profiles in PLNs are quite different between E-IAA(+) and E-IAA(-) mice. They also found genes coding for insulin and for proteins known to be implicated in tissue remodeling and Th1 immunity are highly expressed in PLNs from E-IAA(+) mice. Most of the experiments are properly designed and current findings provide useful information for the understanding of the initial steps of type I diabetes. This reviewer has only minor concerns.

Reviewer’s 1st point:
(1) One of the future prospectives of the current study is to identify non-invasive marker that can predict the onset of diabetes more precisely or earlier than E-IAA. Authors are recommended to list genes that encode soluble proteins and discuss their possible predictive value.

Authors’ response:
As also pointed out by the reviewer, the presence of several secreted proteins was indeed unexpected and represents an interesting future prospective of our data set. In addition as suggested by the reviewer we have grouped several secreted proteins coding genes that have been found to play a role in diabetes, separately under the Functional category “Diabetes”, on Additional Table S5 and several of these genes are discussed in the manuscript.

We believe however that further discussion of the possible predictive values of the secreted proteins for type 1 diabetes, in the absence of experimental data, at this point, would be too speculative. Additional experimental data are necessary as far as the diagnostic potential of the secreted proteins coding genes is concerned, in order to address this point made by the referee.

We hope that our response will satisfy the referee’s suggestion and that the editor will share our point of view concerning this point.

Reviewer’s 2nd point:
(2) One of the six E-IAA(-) samples showed gene expression profile similar to E-IAA(+) samples. Although this reviewer agrees with authors’ explanation, annotations in Fig.2 and 6 are misleading. Authors are recommended to label each lane as either “E-IAA neg” or “E-IAA pos” based on the presence of IAA but not the results of clustering.

Authors’ response:
We thank the referee for this pertinent observation. Indeed we have presented and discussed this discrepancy of sample A36.4 on Table 1 and the corresponding first paragraph on the Results section but we omitted to distinguish this sample on the following figures, in the first submitted version of our manuscript. We have corrected this omission in both Figures 2 and 6 (please see sample A36.4). The hierarchical clustering has been noted according to
phenotypic attribution since it clearly indicates gene expression segregation of the samples used according to the E-IAA phenotype.

**Reviewer’s 3rd point:**
(3) The number of genes coding for extracellular proteins among modulated genes are different between text (p.13, 62 genes) and Figure 4a (60 genes). Authors are asked to explain this discrepancy.

**Authors’ response:**
It might have been overlooked by the referee that the number of genes coding for extracellular proteins is not different between the text (p13, 62 genes) and Figure 4. On Figure 4a are represented the cellular localizations of only the up-regulated genes (60 genes) while on Figure 4b are represented the down-regulated genes (2 genes). In the text we have taken into consideration the total number of modulated genes coding for secreted proteins (up and down regulated), therefore 62 genes. Consequently there is no discrepancy.

**Reviewer’s 4th point:**
(4) The magnifications of Fig.3F and G are different. In addition, the insulin(+) area in PLN (paracortex-medulla) is out of frame in inguinal LN. Authors are recommended to show similar area with the same magnification.

**Authors’ response:**
We have replaced the image of histology for insulin staining of the PLN by an image corresponding to the same magnification and a similar lymph node area as the inguinal LN. Since the inguinal LN was used as negative control and no insulin staining has been observed, regretfully we do not dispose images from sections corresponding to the suggestion of the reviewer.

Even though the reviewer’s comment is scientifically accurate, the quality of the image for the Inguinal LN does not relate to any significant differences for results interpretation. We hope that the reviewer will share our point of view.
Reviewer's report:
1. Is the question posed by the authors well defined? Yes
2. Are the methods appropriate and well described? Yes.
3. Are the data sound? Generally, yes.
4. Does the manuscript adhere to the relevant standards for reporting and data deposition? Yes.
5. Are the discussion and conclusions well balanced and adequately supported by the data? In many instances, the analysis goes well beyond the data presented.
6. Are limitations of the work clearly stated? This is occasionally done.
7. Do the authors clearly acknowledge any work upon which they are building, both published and unpublished? Not to my knowledge.
8. Do the title and abstract accurately convey what has been found? Yes.

Major Compulsory Revisions/ Minor Essential Revisions
There are a number of concerns (minor/major, no specific order), which the authors are advised to consider:
I have read attentively the manuscript submitted by Regnault et al. and found it to address important questions regarding the gene expression basis of autoimmunity and its impact on the immunoregulation of type 1 diabetes (T1D) in NOD mice.
This study explores, with modest success, the relative changes in gene expression occurring at 5 weeks of age, a timepoint where anti-insulin autoAb are detectable and known to discriminate potential sub-phenotypes in disease progression.

Authors’ comment
We thank the reviewer for his comments and his time as well as for giving his attention to the evaluation of this work. We would like to comment however that there is more than a modest success of these experiments. Indeed the obtained data concerning the modulation of a relatively small number of genes (165) in these conditions represented an unexpected result. We did not expect that the comparison of inbred NOD mice that solely differ in our experimental design, by their E-IAA status would present such strong modulation of gene signatures.

Reviewer’s comment:
The authors make some interesting observations indicating that a unique gene expression signature is associated with pre-diabetic NOD mice that positive or not for anti-insulin autoAbs.
The overarching aim of this study was to characterize the differential gene expression underlying this unique sub-phenotype in NOD. The identification of changes in gene activity underlying the onset and passage of one immunoregulatory checkpoint to another more destructive checkpoint is very important is currently under-evaluated in the current literature.
Although innovative in its experimental model and studies well-performed, the
manuscript provides no coherent mechanism for the clinical outcome observed. The major weakness of this study is the lack of mechanistic definition for the described gene signature, and its relationship to autoantibody formation, T cell responses or disease progression.

**Authors’ response**
T1D is known to be a complex disease with several genes implicated in its pathogenesis and environmental factors playing a role. We share the expectations of the reviewer since we also thought that our approach could indeed bring a clearer response about the mechanisms involved in T1D initiation and we hoped that fewer genes would be implicated. Even though this has not been the case, we are convinced that data from our study as well as from similar published reports contribute to a better understanding of this disease. The tremendous information provided by microarray experiments although very valuable, requires usually several additional experiments in order to be validated. Nevertheless our data clearly point towards a mechanism involving tissue damage/degeneration in the aetiology of T1D as it is described in the text after functional annotations of the modulated genes (please see Discussion, section “Tissue regeneration and remodelling”).

**Reviewer’s comment**
Is there a causative role for this gene signature and the onset of autoAb production? Is there a causative role for this gene signature and the break in T cell tolerance which is believed to occur prior to autoAb production?

**Authors’ response**
In the revised version of our manuscript we have taken in consideration all the suggestions and comments made by the reviewer and we have tried to reply to all as best as possible according to our data. Briefly, in response to the above remark, the presence of E-IAA highly suggests that the autoimmune related processes have been initiated in the E-IAA positive animals. This is confirmed also by the modulation of gene profiles related to the selected sub phenotype seen in our data. The modulated genes have been discussed particularly in the Results section (please see Functional significance of the PLN gene signatures). In this section is for example addressed the implication of identified molecules playing a role in NKT cells. We have also addressed issues concerning the local pancreatic tissue early changes in the Discussion section (please see Tissue regeneration and remodelling).

**Reviewer’s comment**
How do the authors exclude the possibility that the described gene signature is merely the consequence of the ongoing autoimmunity/inflammation?

**Authors’ response**
We do not firmly exclude this possibility. Nevertheless hierarchical clustering of gene expression patterns (see Figure 2) according to the selected E-IAA sub phenotype showed a non random distribution of the PLN samples indicating that gene expression profiling corresponded well with the presence or the absence of insulin autoantibodies, omitted one sample (see sample A36.4) which is discussed in the first paragraph of the Results section.

**Reviewer’s comment**
How does this differ between sites of no, low and high inflammation within the same mouse?
Authors’ response
This question raised by the reviewer is a very interesting one. Unfortunately data presented in our manuscript cannot provide an appropriate response to this question, as we have not addressed experimentally this issue. Some speculation from the obtained data certainly can be made but these issues we believe have to first be addressed rather experimentally. The identified genes described herein offer the possibility to design such experiments.

Reviewer’s comment
Is this gene signature simply reflecting the clinical state that is being selected for the disease stratification?

Authors’ response
Yes indeed this could be the case. Other studies using NOD mice, E-IAA positive or negative at 6, 7 and 8 weeks of age for example are required to respond to this question. However it is also possible that at least some of the identified transcripts might indeed play a role in T1D initiation.

Reviewer’s comment
Generally, the study by Regnault et al. article requires several revisions in order to clarify the various issues addressed.

Authors’ response
We have taken in consideration to our best all the comments of this reviewer as well as of reviewer 1, in the revised submitted version of our manuscript and we hope we fulfilled at least some of their expectations for a response.